

## D E S C R I P T I O N

## STABLY PRESERVABLE OXYGEN INFUSION

Various documents will be cited in this specification, and although only the titles are cited here, the entire contents of which are incorporated by

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reference herein for describing the state of the art.

## Technical Field

The present invention relates to a method of preserving an oxygen infusion for a long period of time, an oxygen infusion suitable for a long-term preservation.

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The oxygen infusion of the present invention is widely applicable in the fields of medicine as well as pharmacy. This infusion, as in the case of whole blood transfusion, can be used as it is, or with some additives if necessary, in the field of clinical therapies as a substitute for erythrocytes.

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## Background Art

The current blood transfusion system which infuses human blood into a blood vessel has been encountered with various problems including blood type incompatibility, possibility of infection (hepatitis, HIV and the like) and a too short preservation time period of erythrocytes (only about 3 weeks). Under these circumstances, there has been a great demand for a substitute which can solve these problems, and as one of such substitutes, infusions such as of an

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electrolyte infusion and a colloidal infusion are widely used conventionally.

However, these infusions do not have the most essential function of blood, that is, oxygen-carrying function attributable to the erythrocyte, and therefore it is of a great importance to develop an oxygen infusion (artificial red cells) which can substitute for the oxygen-carrying function of the erythrocyte. Some artificial oxygen infusion has been developed and clinical tests for such oxygen infusions have been advanced. The examples of the oxygen infusions include an aqueous suspension of a perfluorocarbon derivative having high solubility of oxygen; a hemoglobin having a reversible oxygen bonding ability (such as human hemoglobin, bovine hemoglobin or genetically-engineered hemoglobin); an intra-molecular cross-linked hemoglobin; a water-soluble high-molecular conjugated hemoglobin; and an inter-molecular cross linked macromolecular hemoglobin. However, at the same time, it has become evident that various types of side effects arise due to the non-cellular structure of these artificial oxygen infusions.

The followings are possible reasons why hemoglobin (referred to as Hb hereinafter) is inherently contained in the membranes of erythrocytes.

That is:

- 1) To suppress the influence of high viscosity

and/or colloidal osmotic pressure due to a high-concentration Hb solution having a concentration of 12 to 15 g/dl;

2) To seal Hb having high physiological activity within a membrane, thereby suppressing the escape of hemoglobin;

3) To retain each type of phosphoric acid and glycolysis/reduction enzymes, which are used for maintaining the Hb functions, within the same reaction system; and

4) To obtain an advantage of the cell suspension system, which is non-Newtonian fluid exhibiting a characteristic physiological activity within the blood circulatory system (especially, peripheral blood vessels) due to distinctive fluidity thereof.

In consideration of the above-described inherent role of the erythrocyte structure, it is clear that a suspension system of particles encapsulating hemoglobin therein is preferred as the oxygen infusion. In the meantime, it was discovered that a phospholipid, which is a component of living organisms, forms a vesicle structure by itself, and Djordjevich and Miller started studies of hemoglobin vesicles which utilizes liposome made of phospholipid, cholesterol and fatty acid. Thereafter, many organizations have been conducting studies on the hemoglobin vesicle. The use of a hemoglobin vesicle entails advantages such as: 1)

natural hemoglobin can be used as it is; 2) the side effects resulting from hemoglobin can be suppressed; 3) the viscosity, colloidal osmotic pressure and oxygen affinity can be adjusted to arbitrary values, respectively; and 4) the residence (retention) time in circulation system of the living body can be prolonged.

It is conventionally well known to a person skilled in the art that a heme (protoporphyrin IX), which is an oxygen bonding site of hemoglobin, loses its oxygen bonding capability when it escapes from globin. Thus, it has been well recognized that the stereoscopic frame constructed by globin chains plays a significant role and the hydrophobic field formed therein is important. Under these circumstances, a lot of effort has been dedicated for developing a system which can substitute for the functions of globin. The inventors of the present invention studied various types of porphyrin derivatives and have succeeded in synthesizing a lipid heme (lipid-bonded heme):

5,10,15,20-tetrakis[ $\alpha, \alpha, \alpha, \alpha$ -o-(2',2'-dimethyl-20'(2"-trimethyammonioethyl)phosphonatoxy eicosanamido)phenyl]porphyrinato-iron (II) and others, which have a capability of bonding with oxygen reversibly in an aqueous system. In a lipid heme vesicle produced by mixing the above lipid heme together with phospholipid, and then dispersing the resulting mixture in an aqueous phase, the lipid hemes

are embedded in hydrophobic field of a phospholipid membrane and thus suspended and orientated in the membrane. In a lipid heme vesicle in an aqueous suspension system with a uniform particle size, it has been observed that reversible coordination of oxygen is possible as in the case of hemoglobin in a erythrocyte under physiological conditions. Thus, a red-color aqueous system having the same heme concentration as that of blood appeared as the first oxygen infusion manufactured by total synthesis (E. Hasegawa et al., Biochem. Biophys. Res. Commun. vol. 105, 1416 to 1419, 1982). Bioassay was also carried out extensively by administering the lipid hem vesicle into animals. In particular, in the resuscitation test for a canine model of hemorrhagic shock, it was confirmed that the lipid hem had oxygen-carrying capability in accordance with the heme concentration. It was further confirmed that a lipid heme - triglyceride microsphere, prepared by covering the outer surface of an microsphere of a nutritional oil material (such as purified soybean oil or triglyceride) with a lipid heme, has an oxygen carrying capability. Further, synthesized was another oxygen infusion agent which comprises 2-[8-{N-(2-methylimidazolyl)}octanolyloxymethyl]-5,10,15,20-tetrakis[ $\alpha, \alpha, \alpha, \alpha$ -o-pivaloamido]phenylporphyrinato-iron (II) adsorbed in a hydrophobic pocket of human serum albumin or genetically engineered human albumin, the

oxygen infusion agent being referred to as "albumin-heme", hereinafter. Further, it has been confirmed that the albumin-heme has an oxygen carrying capability (E. Tsuchida et al., Bioconjugate Chemistry, vol. 8, 534-538, 1997).

In the present situation of such an oxygen infusion, one of the main issues is the preservation of the oxygen infusion.

There are conventionally known methods for preserving an oxygen infusion, namely, frozen storage and storage in the form of freeze-dried powder. However, it is generally pointed out that the frozen material entails the problem of requiring it to be thawed, which is laborious. On the other hand, the freeze-dried powder requires a lot of time for dissolving in an aqueous solution, and further entails the problem of a complicated operation, such as having to remove the bubbles generated as it dissolves in the solution. Therefore, the frozen storage and freeze-dried powder storage are not preferable.

In addition, the qualities of oxygen infusions deteriorate with time due to the inherent characteristics of heme protein, and therefore it is difficult to preserve them in a stable condition. More specifically, hemoglobin, lipid heme and heme derivatives can reversibly be bonded with oxygen when central iron of heme is a ferrous iron ( $\text{Fe}^{2+}$ ), whereas

when the ferrous iron is oxidized to a ferric iron ( $\text{Fe}^{3+}$ ), they do not have an oxygen binding capability. Further, even a ferrous complex bound with oxygen is gradually oxidized automatically while releasing  
5 superoxide anion ( $\text{O}_2^-$ ), and finally converted to a ferric iron. Thus, the complex loses its oxygen binding capability (for example, hemoglobin becomes methemoglobin). Further, heme protein thus converted to a met-form can easily release free heme and free  
10 ferric iron, which is a concern about causing adverse effects on living body.

Even in the case where the infusion is preserved in a refrigerator to suppress the above-described oxidation by lowering the reaction rate, the amount of  
15 ferric heme gradually increases. In order to solve this problem, there is a conventionally known method of reducing a ferric iron into a ferrous iron by adding a methemoglobin-reducing enzyme system which originally exists in erythrocytes, or an enzyme which can scavenge  
20 active oxygen such as catalase or superoxidedismutase. Also known is a method of maintaining the ferrous iron by binding carbon monoxide (CO) with heme. The affinity of carbon monoxide to hemoglobin or a heme derivative is as high 200 times that of oxygen, and  
25 therefore it is possible to suppress the oxidation to a ferric iron for an extremely long period of time.

However, the above-described method in which a

methemoglobin-reducing enzyme system or an active oxygen scavenger enzyme is added to the oxygen infusion, entails such drawbacks that the enzymatic activity is lowered during a long period of time and thus these enzymes lose their reduction potential. On the other hand, an oxygen infusion which is preserved in a refrigerator under a carbon monoxide atmosphere can not be directly administered into a human body because a great amount of carbon monoxide contained in the oxygen infusion is extremely harmful, and the oxygen bonding potential of the infusion cannot be exhibited unless the carbon monoxides bound with the hem are removed. For this reason, such a transfusion cannot be given as it is to the human body. In addition, in the refrigerator preservation after being converted into an oxy-type, the oxidation to a ferric iron gradually proceeds and eventually the oxygen carrying potential is lowered. The correlation between the oxygen partial pressure of ferrous hemoglobin and the oxidizing rate is well known, and further, it has been experimentally confirmed that the oxidation reaction does not proceed with deoxyhemoglobin (Sakai et al., Bull. Chem. Soc. Jpn., 1994, 1120-1125; Takeoka et al., Bioconjugate Chem., vol. 8, 539-544, 1997).

In addition, even if the oxidation reaction of hemoglobin and heme derivative can be suppressed in any way, the preservation of the oxygen infusion entails



another problem. That is, molecular assembly structures, such as a hemoglobin vesicle, a lipid heme vesicle and a lipid heme-triglyceride microsphere which form the environment of heme, are often unstable since these structures are constructed not with covalent bonds but through molecular interaction forces (such as hydrophobic interaction, electrostatic interaction and hydrogen bonds) acting between molecules of the components. As a result, when such an oxygen infusion is suspended in a saline solution and preserved in a refrigerator, the vesicles are fused with each other to form aggregates of the vesicle population, thereby varying particle diameter thereof. Under these circumstances, there has been a demand for stabilizing the molecular assembly structure of the vesicles. The following is an example of the conventionally known stabilization technique.

That is, it is conventionally known that a polymerizable phospholipid is used as a membrane component of a hemoglobin vesicle or a lipid heme vesicle, and the polymerizable phospholipid is polymerized by  $\gamma$ -ray or ultraviolet ray irradiation to highly stabilizing the structure of the vesicle. In the case utilizing this technique, it is possible to preserve the resultant suspension for a long time by rapidly freezing it with liquid nitrogen. Further, even if the freezing and thawing are repeated for

10 times, a leakage of hemoglobin, a change in the particle diameter or a variation in association-dissociation curve of oxygen cannot be observed (Sato et al., ASAIO Journal, vol. 38, M580 to M584, 1992).

5 In addition, there can be obtained an extremely stable powder by adding a sugar, such as maltose or sucrose, to the above-described suspension system, followed by freeze-drying the system. For example, in the case of hemoglobin vesicle, it was confirmed that an aqueous  
10 suspension of the resultant lyophilized powder showed no leakage of hemoglobin, and no variation in particle diameter thereof, from the physical property analysis carried out on a hemoglobin vesicle which was preserved for 20 weeks at a temperature of 4°C, followed by adding  
15 pure water thereto for re-constituting suspension thereof. This indicates that the hemoglobin vesicle is in substantially the same state as that before the lyophilization (Wang et al., Polymer Adv. Technol., vol. 3, 7-21, 1992).

20 On the other hand, there is a conventionally well-known method of introducing a polyoxyethylene-conjugated lipid onto a surface of a phospholipid vesicle. However, the object of this method is to extend the in-blood retention time of the  
25 vesicle, thereby efficiently transporting an anticancer agent encapsulated therein to a tumor tissue. This method has already undergone clinical trials and the

safety of the method has been fully confirmed.  
Further, it has been empirically confirmed that the  
dynamics of the bloodstream can be improved by  
modifying the surface of a hemoglobin vesicle with  
polyoxyethylene, which can suppress the interaction  
between a hemoglobin vesicle and a plasma protein  
(Sasaki et al., Bioconjugate Chemistry, vol. 8, 23 to  
30, 1997). However, it is not known to utilize the  
polyoxyethylene modification method for the  
preservation of oxygen infusions.

#### Disclosure of Invention

The object of the present invention is to  
establish the technical means which can preserve oxygen  
infusion for a long time at a room temperature on a  
shelf.

According to an aspect of the present invention,  
there is provided a method for preserving an oxygen  
infusion comprising an aqueous suspension containing a  
lipid molecular assembly suspended in an aqueous  
medium, and a heme or heme derivative in the lipid  
molecular assembly, the method comprising:

removing oxygen from the aqueous suspension to  
make the heme or heme derivative into a deoxy-type.

According to another aspect of the present  
invention, there is provided a method for preserving an  
oxygen infusion comprising an aqueous solution of  
albumin-heme, the method comprising:

removing oxygen from the aqueous solution to make a heme or heme derivative contained in the albumin-heme into a deoxy-type; and

5 storing the aqueous solution deprived of oxygen in an inert atmosphere.

According to another aspect of the present invention, there is provided an oxygen infusion suitable for long term preservation, comprising a lipid molecular assembly suspended in an aqueous suspension and a heme or a heme derivative contained in the lipid molecular assembly, wherein

an outer surface of the lipid molecular assembly is modified with polyoxyethylene;

15 the heme or heme derivative is deoxy type; and the oxygen infusion is replenished in an oxygen-impermeable container filled with an inert gas.

According to yet another aspect of the present invention, there is provided a method of producing an oxygen infusion comprising a lipid molecular assembly suspended in an aqueous suspension, and a heme or a heme derivative contained in the lipid molecular assembly, the method comprising:

25 preparing an aqueous suspension of the lipid molecular assembly modified with polyoxyethylene and containing the heme or the heme derivative;

making the hemoglobin or the heme derivative into a deoxy-type by removing oxygen from the aqueous

suspension; and

packing the suspension containing the deoxy-type hemoglobin or heme derivative, in an oxygen-impermeable container which is filled with an inert gas.

5 According to a further aspect of the present invention, there is provided a method of producing an oxygen infusion comprising an aqueous solution of albumin-hem, the method comprising:

10 preparing an aqueous solution containing the albumin-hem;

making a heme or a heme derivative contained in the albumin heme into a deoxy-type by removing oxygen from the aqueous solution; and

15 packing the aqueous solution containing the deoxy-type hem or heme derivative, in an oxygen-impermeable container which is filled with an inert gas.

#### Brief Description of Drawings

20 FIG. 1 is a graph indicating the stability in preservation of a polyoxyethylene-modified deoxy-type hemoglobin vesicle.

#### Best Mode for Carrying Out of the Invention

25 Various documents will be cited in this specification, and although only the title are cited here, the entire contents of which are incorporated by reference herein as supports for the present invention.

In the present invention, the term "lipid molecular assembly" refers to a membrane structure

constructed of molecules such as lipids and/or lipoproteins etc. not through covalent bonds but through interaction (such as hydrophobic interaction, electrostatic interaction and hydrogen bond) acting  
5 between the molecules in an aqueous medium. Typical examples of the lipid molecular assembly are a vesicle or a liposome and a microsphere, and in a broader sense, cell membranes such as erythrocyte membranes are included in the category of the lipid molecular  
10 assembly. Further, a hemoglobin vesicle, lipid heme vesicle and a lipid heme-triglyceride microsphere, as well, are typical examples of the vesicle made of lipid molecular assemblies.

In the specification, the term "heme or  
15 derivatives thereof" encompasses all of the Heme derivatives in which a porphyrin ring of heme is modified with a substituent and has a reversible oxygen-binding potential.

The term "aqueous medium" used in the present  
20 invention includes water and all kinds of aqueous solutions which is physiologically acceptable, such as an electrolyte aqueous solution, a buffer solution, an aqueous protein solution, an aqueous lipid emulsion, blood plasma, a plasma expander (aqueous colloidal  
25 solution such as dextran, hydroxyethyl starch, gelatin or the like) and a combination of any of these.

The inert gas of the present invention means a

chemically inert gas, which is, for example, a rare gas such as helium, argon or neon, or nitrogen. For economical reasons, nitrogen gas is preferable.

5 The following are detailed descriptions for the configuration of the present invention.

Saline suspensions of the following materials were prepared by the methods described in the respective documents, that is, hemoglobin vesicles (Sakai et al., Biotechnology Progress, vol. 12, 119-125, 1996), lipid  
10 heme vesicle (E. Hasegawa et al., Biochem. Biophys. Res. Commun., vol. 105, 1416-1419, 1982), lipid heme-triglyceride vesicle (E. Tsuchida et al., Biochimica Biophysica Acta, vol. 1108(2), p235-256, 1992), and  
albumin heme (E. Tsuchida et al., Bioconjugate  
15 Chemistry, vol. 8, 534-538, 1997). With regard to these aqueous suspensions, it is confirmed that each suspension is in a state where the heme is of a ferrous iron. Then, the suspension is adjusted to have a predetermined components concentration (for example,  
20 hemoglobin concentration of 10 g/dL, heme concentration of 6.2 mM) and oxygen is removed from the suspension. The oxygen removing method operates in the following manner. That is, the suspension is exposed to an oxygen-free nitrogen gas or some other inert gas (such  
25 as of argon or helium), thereby evacuating oxygen dissolved in the suspension. As this operation is carried out, an oxy-type heme is converted into a

deoxy-type heme, to which oxygen is not bound. In practice, the following procedure can be taken. That is, the suspension is charged into a hermetically sealed container such as a glass bottle, through which oxygen cannot permeate, and the inert gas is bubbled within and evacuated from the container to strip the dissolved oxygen from the suspension. In this manner, oxygen remaining dissolved in the infusion can be removed.

The dissolved oxygen concentration can be known with a method of monitoring the oxygen partial pressure while immersing a Clark type oxygen electrode in the suspension, or a method of measuring, by gas chromatography, a gas phase collected from the container, or a method of calculating the ratio between the oxy-type and deoxy-type hemoglobins based on measurements of visible and near infrared spectral absorptions which is characteristic of hemoglobin or heme in the container. Each of thus obtained deoxy-type oxygen infusions can be preserved while being sealed from oxygen, thereby suppressing the oxidation of hemoglobin or heme, or the oxidation of some other components including lipid.

After the above-described oxygen removing operation, in order to further remove a trace amount of oxygen remaining in the solution, an appropriate amount of a thiol (such as homocysteine, acetylcysteine or



glutathione), or a small amount of reductive reagent which reacts with oxygen, such as ascorbic acid and dithionite, may be dissolved into the vesicles or the suspension itself.

5           Each of the resulting deoxy-type oxygen infusions obtained as described above is preserved while being isolated from oxygen. For example, it may be directly sealed in a glass bottle or in an aluminized polyethylene bag or container made of a material having  
10           an extremely low oxygen permeability, such as a polyvinylidene chlorides or ethylene-vinyl alcohol copolymers. Or each agent is sealed in a plastic bag, and the bag is further placed in a container through which oxygen does not permeate. The preservation  
15           temperature should be in a range of  $-20^{\circ}\text{C}$  to  $60^{\circ}\text{C}$ , and more preferably, it should be preserved in a cool and dark place in a range of 4 to  $25^{\circ}\text{C}$ . With the above-described procedure, it is possible to suppress the oxidation of hemoglobin or heme, or the oxidation of  
20           some other component such as lipid, due to oxygen.

          In order to further improve the stability in preservation, it is preferable that, in addition to the above-described oxygen removal, polyoxyethylene should be linked to the surfaces of the molecular assembly  
25           particles in advance. Here, for example, in the case where the above-described molecular assembly is made of lipids as its structural components, it suffices if a

suspension of a lipid having a polyoxyethylene molecule linked thereto (that is, polyoxyethylene lipids) is added at a temperature of 4 to 60°C. The hydrophobic moiety of the polyoxyethylene lipid molecule is  
5 inserted into and fixed on a surface of the molecular assembly particles comprising the lipids, while a hydrophilic polyoxyethylene chain extends into the water phase in an elongated state (Sakai et al., Bioconjugate Chemistry, vol. 8, 23-30, 1997). It  
10 should be noted that the incorporation speed of the polyoxyethylene lipid is faster as the reaction temperature is higher; however the procedure may be carried out at lower temperature. Meanwhile, in the case where a great amount of cholesterol is contained  
15 in the molecular assembly, although no definite phase transition temperature is not specified, the introduction of the polyoxyethylene lipid can be fully conducted even below a phase transition temperature of the phospholipid ingredient. The molecular weight of  
20 the polyoxyethylene chain of the polyoxyethylene lipid may be 1,000 to 20,000 to be sufficient. The incorporation amount is 0.01 to 3 mol% with respect to the total amount of the lipids exposed on the outer surface of each particle, or more preferably, the  
25 incorporation amount should be 0.05 to 0.3 mol%. Examples of the hydrophobic site of the polyoxyethylene lipid include an ethanolamine-type phospholipid,

cholesterol, alkyl-chain-linked glutamic acid, and alkyl-chain-linked lysine. The type of bond between polyoxyethylene and a lipid moiety may be of, for example, an ester bond, urethane bond, amide bond or ether bond. When polyoxyethylene chains are introduced into the surface of each particle, a change in the particle diameter due to the aggregation and fusion of the particles during preservation can be suppressed. On the other hand, in the case of hemoglobin vesicles, it is possible to prevent the leakage of encapsulated elements including hemoglobin from the vesicles.

The effect of the present invention having the above-described structure is as follows. The present invention is designed to suppress the oxidation of hemoglobin or heme derivative in the oxygen infusion by removing oxygen. Due to the effect of the suppression of oxidation, the generation of superoxide anion or hydrogen peroxide can be prevented during preservation, and therefore the oxidation and denaturing of the molecular assemblies which carry hemoglobin or heme derivative. As a result, the physical stability of the molecular assembly particles is improved, and the aggregation of the particles and the change in the particle diameter can be prevented. Therefore, the preservation life of the oxygen infusion comprising molecular assembly particles can be prolonged.

In addition, by introducing polyoxyethylene chain

into the surfaces of molecular assembly particles of hemoglobin vesicles, lipid heme vesicles, lipid heme-triglyceride microspheres, etc., the molecular assembly particles can be further stabilized. In this manner, the change in the particle diameter due to the aggregation and fusion of the particles which may take place during preservation, or the leakage of encapsulated elements including hemoglobin from the particles can be effectively prevented. Therefore, the preservation stability of the oxygen infusion can be further improved.

It should be noted with regard to the present invention that there is a relationship between the oxidation of heme iron from ferrous ion to ferric ion and the instability of the molecular assembly structure, which promote one another mutually. More specifically, superoxide anion ( $O_2^-$ ) and hydrogen peroxide which are generated along the oxidation of heme iron, as well as ferri-hemoglobin thus created, serve to oxidize the structural components of the molecular assemblies, thereby promoting the destruction of the molecular assemblies. On the other hand, the destruction of the molecular assemblies can deteriorate the circumstances where heme irons exist, thereby promoting the oxidation of the heme. The present invention focuses on this point and aims to suppress the oxidation of hemoglobin and a heme derivative, and

to stabilize molecular assemblies serving as carriers for hemoglobin and the heme derivative, at the same time. In this manner, it becomes possible to store the oxygen infusion at room temperature on a shelf, which cannot be conventionally achieved.

It should be noted that the oxygen infusion which uses an albumin-heme is stable in the state of solution, and therefore a relatively high stability can be achieved even though the modification by polyoxyethylene is not employed. However, with the modification by polyoxyethylene, the stability can be further improved. When this modification is combined with the preservation in an oxygen-free state, the conversion into met-type can be prevented, and thus the preservation stability can be remarkably improved, as in the above-described case.

Thus obtained deoxygenated-type oxygen infusions can be preserved for a long term. Therefore, when the oxygen infusion is kept in stock regularly in sections of clinical facilities, ambulances and remote areas where no medical facilities are located, the oxygen infusion can be administered into the patient's body immediately when needed. The deoxy-type oxygen infusion, when exposed to atmosphere, is bound with oxygen to become an oxy-type. On the other hand, even if the oxygen infusion is administered into a vein directly in the form of deoxy-type, it binds with

oxygen immediately when it first passes the lung to become an oxy-type, and then releases oxygen in peripheries.

Next, the present invention will now be described in more detail by way of practical examples.

#### Example 1

Pyridoxal 5'-phospholic acid was added to a high-purity stroma-free carbonyl hemoglobin (HbCO) solution (40 g/dL) obtained by purification of human erythrocytes such that the amount of Pyridoxal 5'-phospholic acid became 3 times in molar as much as that of hemoglobin. Then, homocysteine was further added to the mixture to become a concentration of 5 mM, and then pH of the resultant mixture was adjusted to 7.4 with use of 1M- $\text{Na}_2\text{CO}_3$ . Thus obtained mixture was filtrated through FM Microfilter (a product of FUJI FILM) having a pore diameter of 0.22  $\mu\text{m}$  using Remolino (a product of Millipore), and a stock hemoglobin solution was obtained. After that, a mixed lipid powder, Presone PPG-I (a mixture of phosphatidylcholine/cholesterol/phosphatidylglycerol) was added to the solution little by little until the lipid concentration became 4.5% by weight, and then the resultant mixture was stirred at a temperature of 4°C overnight, thus obtaining hemoglobin-containing vesicles. With an extrusion method, the diameter of particles and the total layers of these vesicles were controlled. Eventually, the filtration

was carried out two times by using FM Microfilter (having a pore diameter of 0.22  $\mu\text{m}$ ). Thus obtained hemoglobin-containing multi-layered vesicles were diluted with a physiological saline to form suspension, and then, the resultant suspension was subjected a ultracentrifugal separation (50,000g, 40 min). After that, the supernatant hemoglobin solution was removed by suction, and then, the resultant hemoglobin vesicles are re-suspended in a physiological saline.

Then, a polyoxyethylene-conjugated lipid, N-(monomethoxypolyoxyethylene-carbamyl)distearoylphosphatidyl-ethanolamine, dissolved in a physiological saline (molecular weight of polyoxyethylene chain was 5300) was supplied dropwise to the above-prepared suspension, in an amount equivalent to 0.3 mol% of the lipids exposed on the outer surface of each vesicle. The resultant was stirred at 25°C for two hours, and then at 4°C overnight, thus modifying the surface of each hemoglobin vesicle with polyoxyethylene.

The hemoglobin vesicle suspension (0.5g/dL, 200 mL) was put in a cylindrical flask, which in turn was loaded in a rotary evaporator, and thus the flask was rotated (56 rpm). Then, on a liquid membrane formed by this operation, visible light was irradiated using a halogen lamp (500W) under an oxygen stream condition (1 L/min) for 3 minutes. In this manner,

carbon monoxide -bound hemoglobin (HbCO) was converted into oxyhemoglobin (HbO<sub>2</sub>) by way of ligand exchange.

Thus obtained suspension was subjected to a ultracentrifugal separation (50,000g, 60 min) so as to

5 sediment hemoglobin vesicle particles, and then, physiological saline as the external aqueous phase was removed.

Then, the resultant hemoglobin vesicles were re-suspended by adding the phosphate buffered physiological saline to the particles.

Thus obtained suspension was filtrated with a 0.45  $\mu$ m-filter, Dismic-25 (a product of ADVANTEC) after setting the hemoglobin

10 concentration to 10 g/dL. In this manner, polyoxyethylene-modified hemoglobin vesicles were obtained.

15 30 mL of the suspension of the polyoxyethylene-modified hemoglobin vesicles described above was received in a 100 mL vial and sealed therein. Then, a nitrogen gas, which was filtered through a sterile disk filter and saturated with water vapor, was introduced

20 to the vial and bubbled within the vesicle suspension in order to remove the dissolved oxygen. The oxygen partial pressure within the system was monitored with

use of a Clark type oxygen electrode (Oxygen Partial Pressure Measuring Apparatus, = Po<sub>2</sub>-100, Inter

25 Medical), and it was observed that the oxygen partial pressure was decreased to 1 Torr. Thus, it was determined that oxyhemoglobin was converted into



deoxyhemoglobin by the procedure described above.

Thus obtained oxygen infusion according to the present invention was subjected to preservation test. Preservation conditions used here were preservation in  
5 a refrigerator (4°C), room temperature preservation (23°C), and preservation in an incubator (40°C). With regard to samples for these conditions, the following measurements were carried out for one year and the measured results were compared with the sample before  
10 these preservations.

(1) 30  $\mu$ L of each sample was diluted by 100 folds with physiological saline and then each dilution was subjected to measurement in terms of ultraviolet visible absorption spectrum from 300 to 900 nm by using  
15 a 1-mm cuvette at room temperature. As compared with the samples before the preservation, the presence/absence of development of a new absorption peak, the shift of the wavelength where a Q-band peak and the like were studied.

(2) The presence/absence of a sediment formation in a respective sample was visually monitored with naked eyes. 30  $\mu$ L of each sample was diluted by 10 folds with physiological saline, and then, subjected to measurement in terms of absorbance at 900 nm by  
25 using a 1-mm cuvette at room temperature. The absorbance of the physiological saline at 900 nm was subtracted as a reference from the measured value, and

thus obtained value was taken as the turbidity of the respective sample.

5           (3) About 0.2 mL of each sample was diluted with a phosphate buffered saline (PBS) by 200 folds, and then, subjected to ultracentrifugal separation (100,000g, 15 min). After that, the supernatant liquid of each sample was examined for quantitative analysis of hemoglobin, and thus the presence/absence of hemolysis was determined.

10           (4) The distribution of the particle diameters was measured by a dynamic light-scattering photometry at a temperature of 25°C using Sub-micron Particle Analyzer Model N4-SD (Coulter Corporate Communications).

15           (5) An oxygen association/dissociation curve was measured with use of Hemox-Analyzer (TCS Model Products Co.), and from the analysis, the oxygen affinity ( $P_{50}$ ), the oxygen-transporting efficiency (OTE) and the Hill number were calculated.

20           (6) In order to study the decomposition of the lipids, about 0.2 mL of each sample was lyophilized and the lipids were extracted using  $\text{CHCl}_3$ . The measurement was conducted by two-dimensional thin layer chromatography (silica gel plate) using, as developing  
25           mediums, chloroform/methanol/28% ammonia = 13/7/1 (in volume ratio) and chloroform/acetone/methanol/acetic acid/water = 10/4/2/2/1 (in volume ratio).

(7) About 0.2 mL of each sample was lyophilized and the membrane components were extracted with about 1 mL of  $\text{CDCl}_3$ , followed by filtration with a filter. Then, the resultant sample was measured in terms of  $^1\text{H}$ -NMR spectrum (JNM-LA500, Nihon Denshi). On the other hand, in order to remove polyoxyethylene chains dissociated into the external aqueous phase, about 0.2 mL of each sample was diluted with PBS by about 200 folds and the supernatant liquid was removed by a ultracentrifugal separation (100,000g, 15 min). After the sediments was re-suspended with PBS, the resultant was freeze-dried, and then the membrane components were extracted using about 1 mL of  $\text{CDCl}_3$ , followed by a filtration with a filter. Then, the resultant was measured in terms of  $^1\text{H}$ -NMR spectrum. The peak (B) which is assigned to the methylene protons of polyoxyethylene chain in polyoxyethylene lipid appeared at  $\delta$  : 3.63 ppm, whereas the peak (A) which is assigned to choline methyl proton of phosphatidylcholine appeared at  $\delta$  : 3.39 ppm. Supposing that the ratio between the number of protons in the peak (A) to that in the peak (B) is equal to the integral ratio of B/A, the incorporation ratio of polyoxyethylene chains was calculated by way of the following formula:

$$B/A(\text{after}) \div B/A(\text{stock}) \times 100$$

wherein

B/A(after) is the Integral ratio B/A after removal of external water phase); and

B/A(stock) is the integral ratio B/A of stock solution.

5           FIG. 1 shows changes of various physical property values of hemoglobin vesicle suspension during the time course of the preservation. In any of the samples, appearance of a new peak at 630 nm characteristic to the methemoglobin in the ultraviolet visible absorption  
10 spectrum, a change in absorbance of Q-band or Soret band, or shift of wavelength was not observed during a preservation period for 1 year. Further, no hemolysis was confirmed or no dissociated fatty acid was observed in the two-dimensional thin layer chromatography. In  
15 any of the samples, after six months of preservation, no sediment due to aggregation was observed, and the particle diameters or turbidity was not substantially changed. Further, after preserving for six months at 40°C, the polyoxyethylene chain incorporation was  
20 maintained only to a decrease of about 7% as compared to that before the preservation. The decrease of P<sub>50</sub> was as small as 5.5 Torr as compared to that before the preservation, even after preserving for six months at 40°C. With such a small degree of decrement, it was  
25 determined that the oxygen transporting function of hemoglobin vesicle was not affected. However, in the case of a preservation of one year at 40°C, a

decomposition of a lipid and a decrease of  $P_{50}$  to 43 Torr were observed. In any of the samples, the initial rate of conversion into methemoglobin after preservation was decreased, and became less than 1% after one month of preservation. This is because that the oxidized methemoglobin was reduced by homocysteine. From the above observations, it was determined that the hemoglobin vesicle whose surface was modified with polyoxyethylene chain can be preserved for six months at 40°C, or one year at 23°C, on a shelf under a nitrogen atmosphere.

#### Example 2

A suspension of hemoglobin vesicles which are not modified with polyoxyethylene was prepared in a similar manner to that of Example 1, and the suspension was received in a vial and sealed therein. Then, a nitrogen gas, which was filtered with a sterile disk filter and saturated with water vapor, was introduced to the vial and was bubbled within the vesicle suspension in order to remove the dissolved oxygen completely. The oxygen partial pressure within the system was monitored by using Oxygen Partial Pressure Measuring Apparatus ( $PO_2$ -100, a product of Inter Medicals), and it was observed that the oxygen partial pressure was decreased to 2 Torr. Thus, it was determined that with the above-described procedure, oxyhemoglobin was converted into deoxyhemoglobin.

Thus obtained oxygen infusion according to the present invention was subjected to preservation test. Preservation conditions used here were preservation in a refrigerator (4°C), room temperature preservation  
5 (23°C), and preservation in an incubator (40°C). With regard to samples for these conditions, the following measurements were carried out for six months and the measured results were compared with the sample before these preservations. That is, the presence/absence of  
10 a sediment formation in the sample in each case was visually observed with naked eyes. 30 µL of each sample was diluted by 10 folds with physiological saline, and then, subjected to measurement in terms of absorbance at 900 nm using a 1-mm cuvette at room  
15 temperature. The absorbance of the physiological saline at 900 nm was subtracted as a reference from the measured value, and thus obtained value was taken as the turbidity of the respective sample. The measurement of the distribution of particle diameters  
20 was conducted by a dynamic light-scattering method using Sub-micron Particle Analyzer Model N4 SD (Coulter Corporate Communications) at a temperature of 25°C.

An increase in the methemoglobin content was not at all observed, and it became substantially constant  
25 after one month of preservation. With regard to the increase in the particle diameter, it increased about 8% after one week of preservation and a small amount of

sediment formed by aggregation was observed. However, each sample was still in a usable condition. By contrast, in the case where oxygen was not removed, sediment was formed as early as one week of preservation to such a degree that the infusion cannot be used. Therefore, it can be understood that the removal of oxygen contributed also to the stabilization of hemoglobin vesicles.

However, from the comparison with the results of Example 1, it was found that the particle diameter drastically increased during the preservation in each sample. Such results indicate that the modification of the surfaces of hemoglobin vesicles with polyoxyethylene, and the preservation of the agent under an oxygen-free condition, interact synergistically with each other, thereby achieving a further significant preservation stability.

### Example 3

A polyoxyethylene-modified hemoglobin vesicle suspension (the molecular weight of polyoxyethylene: 2000) was prepared in a similar manner to that of Example 1. Thus obtained deoxy-form was transferred into an aluminum bag (Aluminized polyethylene bag, a product of GL Sciences, Inc.) under a nitrogen atmosphere, in order to isolate it from oxygen. Thus obtained infusion were preserved under conditions of preservation in a refrigerator (4°C), room temperature

preservation (23°C), and preservation in an incubator (40°C). With regard to each sample for these conditions, the same measurements as those of Example 1 were conducted for one year. The results obtained here were similar to those of Example 1.

#### Example 4

A polyoxyethylene-modified hemoglobin vesicle suspension (50 mL) was prepared in a similar manner to the preparation method employed in Example 1 except that homocystein used in Example 1 was replaced by glutathione and the molecular weight of polyoxyethylene chain of the polyoxyethylene lipid was adjusted to 10,000. Thus prepared suspension was received in a cylindrical flask (2L), which was loaded in a rotary evaporator and rotated (60 rpm), thus creating a liquid membrane of the hemoglobin vesicle suspension. A nitrogen gas was put through (1.0 L/min) the liquid membrane in order to remove oxygen therefrom. Then, it was confirmed using a near-infrared region noninvasive oxygen monitor (Model OM-200, a product of Shimazu Corporation) that 98% or more of the entire hemoglobin was deoxy hemoglobin. Thus obtained resultant was sealed in a refrigeration pack, Cryocyte (a product of Baxter) and further sealed in a aluminum can in order to block off the penetration of oxygen. Thus obtained infusions were subjected to preservation test, in which they were preserved under conditions of preservation in



a refrigerator (4°C), room temperature preservation (23°C), and preservation in an incubator (40°C). With regard to each sample for these conditions, the same measurements as those of Example 1 were conducted for one year. The results obtained here were similar to those of Example 1.

#### Example 5

A lipid heme vesicle suspension was made of 5,10,15,20-tetrakis[ $\alpha, \alpha, \alpha, \alpha$ -o- {2',2'-dimethyl-20'(2"-trimethylammonioethyl)phosphonatoxyeicosanamido}-phenyl]porphyrinato-iron(II) (lipid heme)/1-stearylimidazole/dipalmitoyl phosphatidyl choline/cholesterol/polyoxyethylen-conjugated phospholipid which is N-(monomethoxypolyoxyethylenecarbamyl) diphosphatidyl ethanolamine, in a molar ratio of 1/3/40/20/2.5. Here, the average molecular weight of the polyoxyethylene chains was adjusted to 5000. To the suspension, physiological saline was added to prepare a solution having a lipid heme concentration of 5 mM. The solution was subjected to the extrusion method described in Example 1 so as to control the particle diameter, and then sealed into a glass container with addition of 6 mM of ascorbic acid. Then, a nitrogen gas was put through the solution by the same method as in Example 1. As a result, ferric iron hemes were all

reduced to ferrous iron hemes and the oxygen partial pressure was reduced to low as 3 Torr; therefore substantially all of the vesicles in the container became deoxy-type lipid heme vesicles. Thus obtained  
 5 infusion was preserved at room temperature for three months, and the analysis thereof did not show any indication of increase in the amount of ferric iron heme. Further, the particle diameter was  $105 \pm 21$  nm before the preservation, whereas after the  
 10 preservation, it was  $107 \pm 28$  nm, exhibiting no substantial change. A significant increase in turbidity was not observed.

#### Example 6

For the preparation of a lipid heme - triglyceride  
 15 microsphere suspension, a soybean oil ([soybean oil]/[heme] = a ratio of 2 to 4 by weight) was added to 5,10,15,20-tetrakis[ $\alpha, \alpha, \alpha, \alpha$ -o-(2',2'-dimethyl-20'(2"-  
 trimethylammonioethyl)phosphonatoxyeicosanamido)phenyl]  
 20 porphyrinato-iron(II) (lipid heme)/1-stearylimidazole (at a ratio in molar of 1/2.5), and further a 2%-glyceline aqueous solution was added thereto. Then, the mixture was subjected to supersonic agitation in a water bath under a nitrogen atmosphere, thus obtaining the  
 25 suspension. To the suspension, a polyoxyethylene-conjugated lipid having an average molecular weight of 2000, N-(monomethoxypolyoxyethylenecarbamyl)

dipalmitoylphosphatidyl ethanolamine, was added at a ratio of 0.02 mol% with respect to the lipid heme, so as to modify the lipid heme - triglyceride microsphere with polyoxyethylene. 180 mL of thus obtained suspension was then sealed into a 200 mL-glass container with a slightly excessive amount of ascorbic acid added therein. Then, the bubbling with a nitrogen gas was performed by the same method as in Example 1, and thus the oxygen partial pressure was reduced to low as 2 Torr. Thus, deoxy-type lipid heme - triglyceride microspheres were obtained. The resultant suspension was preserved at room temperature for four months, and the analysis thereof did not show any indication of increase in the amount of ferric iron heme. Further, the particle diameter was  $85 \pm 25$  nm before the preservation, whereas after the preservation, it was  $86 \pm 28$  nm, exhibiting no substantial change.

#### Example 7

Albumin-heme was prepared from a heme derivative, i.e., (2-[8-{N-(2-methylimidazolyl)} octanoyloxymethyl]-5,10,15,20-tetrakis( $\alpha, \alpha, \alpha, \alpha$ -o-pivalamido)phenylporphyrinato iron(II)), and human serum albumin by the method described in the aforementioned document (E. Tsuchida et al., Bioconjugate Chemistry, vol. 8, 534-538, 1997), the content of which is incorporated herein by reference. After confirming that the ferric iron hemes are bound with oxygen, the

resultant albumin - heme solution was sealed into a glass container. Then, a nitrogen gas was put through the solution by the same method as in Example 1, and thus the oxygen partial pressure was reduced to low as 3 Torr. Thus, the deoxy-type albumin-hemes were obtained. The resultant albumin-heme solution was preserved at 20°C for five months, and the analysis thereof did not show any indication of increase in the amount of ferric iron heme. Further, an increase in the amount of insoluble matter was not observed.

#### Comparative Example 1

A polyoxyethylene-modified hemoglobin vesicle suspension was prepared in a similar manner to the preparation method employed in Example 1. The oxygen partial pressure was adjusted to the same as that of atmosphere, that is, 149 Torr, under a sterile atmosphere. Thus obtained suspension was sealed in a vial, which was preserved in a incubator (40°C) without removing oxygen therefrom, and therefore, in the form of oxyhemoglobin. During the preservation, the rate of conversion into methemoglobin was measured from the ultraviolet visible light absorption spectrum at 1, 4 and 24 hours later. As the time elapsed during the preservation, methemoglobin content was increased from 2.7%, which was the value before the preservation, to 5% after one hour of preservation, to 12% after four hours of preservation and to 36% after 24 hours of

preservation.

The above-described results indicate that even a polyoxyethylene-modified hemoglobin vesicle suspension exhibits a significant increase in the amount of methemoglobin when oxygen is not removed. Therefore, in such a case, a preservation stability similar to that of the present invention cannot be obtained.

## C L A I M S

1. A method for preserving an oxygen infusion comprising an aqueous suspension containing a lipid molecular assembly suspended in an aqueous medium, and  
5 a heme or heme derivative in the lipid molecular assembly, the method comprising:

removing oxygen from the aqueous suspension to make the heme or hem derivative into a deoxy-type.

2. The method for preserving an oxygen infusion  
10 according to claim 1, further comprising:

storing the aqueous suspension after being removed of oxygen in an inert atmosphere.

3. The method for preserving an oxygen infusion according to claim 1, further comprising:

15 modifying the molecular assemblies with polyoxyethylene.

4. The method for preserving an oxygen infusion according to claim 1, wherein the molecular assembly is selected from the group consisting of hemoglobin  
20 vesicle, lipid heme vesicle and lipid heme - triglyceride microsphere.

5. The method according to claim 1, wherein the removal of oxygen is performed by means of gas exchange with an inert gas.

25 6. A method for preserving an oxygen infusion comprising an aqueous solution of albumin-heme, said method comprising:

removing oxygen from the aqueous solution to make a heme or heme derivative contained in the albumin-heme into a deoxy-type.

5        7. The method according to claim 6, wherein the removal of oxygen is performed by means of gas exchange with an inert gas.

8. The method according to claim 6, further comprising:

10        storing the aqueous suspension after being removed of oxygen in an inert atmosphere.

9. An oxygen infusion comprising a lipid molecular assembly suspended in an aqueous suspension and a heme or a heme derivative contained in the lipid molecular assembly, wherein

15        an outer surface of the lipid molecular assembly is modified with polyoxyethylene;

the heme or heme derivative is deoxy type; and

the oxygen infusion is replenished in an oxygen-impermeable container filled with an inert gas.

20        10. The oxygen infusion according to claim 9, further comprising a physiologically acceptable reducing agent.

11. An oxygen infusion comprising an aqueous solution containing albumin-heme, wherein

25        a heme or heme derivative contained in the albumin-heme is deoxy type; and

said oxygen infusion is replenished in a oxygen-

impermeable container filled with an inert gas.

12. The oxygen infusion according to claim 11, further comprising a physiologically acceptable reducing agent.

5        13. A method of producing an oxygen infusion comprising a lipid molecular assembly suspended in an aqueous suspension, and a heme or a heme derivative contained in the lipid molecular assembly, the method comprising:

10        preparing an aqueous suspension of the lipid molecular assembly modified with polyoxyethylene and containing the heme or the heme derivative;

      making the hemoglobin or the heme derivative into a deoxy-type by removing oxygen from the aqueous  
15        suspension; and

      packing the suspension containing the deoxy-type hemoglobin or heme derivative, in an oxygen-impermeable container which is filled with an inert gas.

20        14. The method according to claim 13, wherein the removal of oxygen is performed by means of gas exchange with an inert gas.

      15. A method of producing an oxygen infusion comprising an aqueous solution of albumin-hem, the method comprising:

25        preparing an aqueous solution containing the albumin-hem;

      making a heme or a heme derivative contained in



the albumin heme into a deoxy-type by removing oxygen from the aqueous solution; and

5            packing the aqueous solution containing the deoxy-type hem or heme derivative, in an oxygen-impermeable container which is filled with an inert gas.

16. The method according to claim 15, wherein the removal of oxygen is performed by means of gas exchange with an inert gas.

## A B S T R A C T

There is provided a method for preserving an oxygen infusion comprising an aqueous suspension containing a lipid molecular assembly suspended in an aqueous medium, and a heme or heme derivative in the lipid molecular assembly, the method comprising making the hemoglobin or the heme derivative to a deoxy-type by removing oxygen from the suspension, and storing the aqueous suspension after being removed of oxygen in an inert atmosphere.